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20 Running title: Menopause, training and the apoM/S1P axis

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22

## 23 Abstract

24 **Objective:** The axis of apolipoprotein M (apoM) and sphingosine-1-phosphate (S1P) is of importance to  
25 plasma lipid levels, endothelial function, and development of atherosclerosis. Menopause is accompanied by  
26 dyslipidemia and an increased risk of atherosclerosis, which can be lowered by exercise training. The aim of  
27 this study was to explore if effects of menopause and training are paralleled by changes in the apoM/S1P  
28 axis.

29 **Methods:** Healthy, late premenopausal (n=38, age 49.2±2) and recent postmenopausal (n=37, age 53.3±3)  
30 women from the Copenhagen Women Study participated in a three-month, aerobic high-intensity exercise  
31 intervention.

32 **Results:** Before training, plasma apoM was higher in postmenopausal (1.08±0.2 µmol/l (mean±SD))  
33 compared to premenopausal (0.82±0.2 µmol/l) women (p<0.0001). Plasma S1P was similar in the two  
34 groups (0.44±0.1 and 0.46±0.1 µmol/l, respectively). Hence, the pre-training S1P/apoM ratio was 26% lower  
35 in postmenopausal than premenopausal women (p<0.0001). After the training program, plasma apoM  
36 increased from 0.82±0.2 to 0.90±0.3 µmol/l in premenopausal women and from 1.08±0.2 to 1.16±0.3 µmol/l  
37 in postmenopausal women (p<0.05). Plasma S1P increased from 0.44±0.1 to 0.47±0.1 µmol/l in  
38 premenopausal women and from 0.46±0.1 to 0.48±0.1 µmol/l in postmenopausal women (p<0.05).

39 **Conclusions:** The results suggest that menopause is accompanied by higher plasma apoM but not S1P  
40 concentrations, and that exercise training increases plasma apoM and S1P in healthy middle-aged women  
41 irrespective of menopausal status.

42

## 43 Keywords

44 Apolipoproteins, lipoprotein metabolism, lipids, sphingolipids, atherosclerosis, menopausal transition,  
45 cardiovascular training

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48 **New & Noteworthy**

49 The ApoM/S1P complex is involved in maintaining a healthy endothelial barrier function. Our study is the  
50 first to show how menopause affects apoM/S1P axis. The results suggest that the menopause is accompanied  
51 by higher plasma apoM but not S1P concentrations. Secondly, the study is also the first to show that exercise  
52 training increases both apoM/S1P in women irrespective of menopausal status.

## 53 **Introduction**

54 Menopause is associated with an elevated risk of developing endothelial dysfunction (33) and metabolic  
55 syndrome, including dyslipidemia and atherosclerosis (7). Long-term hormone therapy is not generally  
56 recommended for postmenopausal women due to increased risk of cancer and athero-thrombotic disease  
57 (32). Currently, there is a lack of mechanistic understanding of changes that cause dyslipidemia,  
58 atherosclerosis, and endothelial dysfunction in postmenopausal women.

59 Apolipoprotein M (apoM) is a lipocalin mainly bound to plasma high density lipoprotein (HDL)  
60 particles (11). ApoM has an important role in protecting the endothelial barrier function (8, 12) and affects  
61 several potential anti-atherogenic pathways, such as reverse cholesterol transport (10, 19), formation of pre $\beta$ -  
62 HDL (10, 34, 47), and removal of reactive oxygen species (10, 18). Moreover, variations in the apoM gene  
63 are associated with risk of cardiovascular disease (CVD) and altered plasma lipids (5). Further investigations  
64 are however needed to clarify the exact role of apoM in atherosclerosis and dyslipidemia.

65 The bioactive lipid sphingosine-1-phosphate (S1P) is carried by apoM (12). ApoM-containing HDL  
66 carries ~65 % of plasma S1P, while albumin carries ~35 % (27, 35). Hence, apoM is a chaperone, and its  
67 biological effects are likely provided by S1P (36, 40). S1P acts through five G-protein-coupled receptors  
68 (S1P<sub>r1-5</sub>) (24), affecting diverse processes such as protection of the endothelial barrier (8, 12, 45), regulation  
69 of angiogenesis (16), promotion of lymphocyte trafficking (39), and activation of endothelial nitric oxide  
70 synthase (eNOS) (23). Accordingly, S1P has been implicated in several diseases, including inflammatory  
71 diseases and atherosclerosis (30).

72 Exercise training decreases CVD risk by effects on endothelial function (21) and plasma lipids,  
73 including in postmenopausal women (4, 28, 42). This finding is of particular interest as the menopausal  
74 transition is accompanied by increased prevalence of dyslipidemia (7, 14, 15, 25), and endothelial  
75 dysfunction (33). The apoM/S1P axis is associated with endothelial function (8, 12) and plasma lipids (5),  
76 which are all influenced negatively by the menopausal transition and can be improved with exercise training.  
77 The effect of menopause and exercise training on the apoM/S1P axis itself is unknown, and further  
78 investigation is needed in order to understand if apoM and S1P are involved in menopausal and exercise-  
79 induced changes. This has prompted the present study on how plasma apoM and S1P concentrations are

80 affected by exercise training in early postmenopausal compared with late premenopausal women, as well as  
81 how apoM and S1P concentrations differ in pre-and postmenopausal women.

82

83

## 84 **Materials and methods**

85 The data presented in this study are part of the Copenhagen Women Study (31, 37), which is an  
86 interdisciplinary study on the effects of exercise training in the late premenopausal and early postmenopausal  
87 phase. The study was approved by the Ethics Committee in the capital region of Denmark (protocol number:  
88 H-1-2012-150) and hosted institute. All participants were given informed consent at time of inclusion, and  
89 the study was conducted in accordance with the guidelines of the Declaration of Helsinki.

90 *Participants* - In the present study, we used samples from 38 premenopausal women and 37 postmenopausal  
91 women from the Copenhagen Women Study (31, 37). All included participants were healthy non-smokers  
92 with no excessive alcohol intake and a body mass index of 18.5-30 kg/m<sup>2</sup>. The premenopausal women had  
93 regular menstrual cycles and were not using hormonal contraceptives. The postmenopausal women had not  
94 experienced a menstrual cycle for at least 1 year and were not receiving hormone therapy. All women were  
95 physically active less than 2 hours per week prior to the training intervention.

96 *Study design* - As described previously (31, 37), all women underwent testing before and after three months  
97 of high-intensity exercise training on a cycling ergometer for one hour three times per week. The training  
98 sessions were supervised by instructors, and exercise intensity was monitored and increased gradually during  
99 the three-month period. As described by Nyberg et al (37), the heart rate was at 71-95% of the maximal heart  
100 rate almost 90% of the time. On test days, all women were fasting and had not exercised for 24 hours.

101 *Plasma lipids* - Blood samples taken with a BD Vacutainer system (Becton-Dickinson, Plymouth, UK) were  
102 analyzed at the Department of Clinical Biochemistry at Rigshospitalet, Denmark. Plasma aliquots were used  
103 for analysis of total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-  
104 cholesterol (LDL-C), and triglycerides with enzymatic absorption photometry (Cobas 8000, c702 module, F.  
105 Hoffmann-La Roche Ltd., Rotkreuz, Switzerland). Other blood samples for apoM and S1P measurements  
106 were centrifuged for 5 minutes at 4000g and stored at -80°C. Blood samples for S1P measurements were  
107 placed on ice immediately after collection.

108 *Plasma apoM* - ApoM was measured with ELISA as described by *Bosteen et al.* with intra and inter assay  
109 coefficients of variation of 3.2% and 7.9%, respectively (6). Plasma for measurement of apoM was available  
110 for 38 premenopausal women (38 pre-training samples and 36 post-training samples) and 37 postmenopausal  
111 women (37 pre- and post-training samples). For the two-way repeated measures ANOVA, we could only  
112 include subjects with paired values obtained before and after exercise training (pre-menopause n=36;  
113 postmenopause n=37).

114 *Plasma S1P* - S1P was measured with an HPLC-based method as described by *Christoffersen et al.* (12).  
115 Plasma for measurement of S1P was available for 37 premenopausal women (36 pre-training samples and 31  
116 post-training samples) and 35 postmenopausal women (32 pre-training samples and 33 post-training  
117 samples). For two-way repeated measures ANOVA, we could only include subjects with paired values  
118 obtained before and after exercise training (premenopause n=30; postmenopause n=30).

119 *Statistical Analysis* - Data were analyzed using GraphPad Prism 4 software. The significance level (alpha)  
120 was set at  $p < 0.05$ . The effects of menopausal status and exercise training were assessed using two-way  
121 repeated measures ANOVA; only women with measurements before and after the training intervention were  
122 included in the two-way repeated measures ANOVA. Correlations were assessed using Pearson correlation  
123 analysis. Normally distributed data are presented as mean $\pm$ SD. Data that are not normally distributed are log-  
124 transformed and presented as median (25-75 percentile). After the log-transformation, all data were normally  
125 distributed.

126



## 127 **Results**

128 *Characteristics of the participants* - Before the training intervention, the premenopausal women were  $49.2 \pm 2$   
129 years old. The postmenopausal women were  $53.3 \pm 3$  years old and  $3.1 \pm 1$  years past their final menstruation.

130 *Effects of menopausal status* - Before training, the plasma apoM concentration was 32 % higher in the  
131 postmenopausal than in the premenopausal women ( $1.08 \pm 0.2$   $\mu\text{mol/l}$  and  $0.82 \pm 0.2$   $\mu\text{mol/l}$ , respectively)  
132 ( $p < 0.0001$ ) (Figure 1A). The plasma S1P concentration was similar in the two groups ( $p > 0.05$ ) (Figure 1B).  
133 Hence, the S1P/apoM ratio was 26 % lower in postmenopausal than premenopausal women before training  
134 ( $0.45 \pm 0.1$  and  $0.61 \pm 0.2$ , respectively) ( $p < 0.001$ ) (Figure 2A). In accordance with previous findings from the  
135 Copenhagen Women Study (40), plasma TC ( $p < 0.0001$ ), HDL-C ( $p < 0.001$ ), and LDL-C ( $p < 0.01$ )  
136 concentrations were higher in this subset of postmenopausal compared with premenopausal women, while  
137 the plasma triglyceride concentration was similar in the two groups ( $p > 0.05$ ) (Table 1). The plasma  
138 apoM/HDL-C ratio was also similar in the two groups ( $p > 0.05$ ) (Figure 2B).

139 *Effects of exercise training* - The three-month high-intensity training intervention increased the plasma apoM  
140 concentration from  $0.82 \pm 0.2$  to  $0.90 \pm 0.3$   $\mu\text{mol/l}$  in premenopausal women and from  $1.08 \pm 0.2$  to  $1.16 \pm 0.3$   
141  $\mu\text{mol/l}$  in postmenopausal women ( $p < 0.05$ ) (Figure 1A). The plasma S1P concentration increased from  
142  $0.44 \pm 0.1$  to  $0.47 \pm 0.1$   $\mu\text{mol/l}$  in premenopausal women and from  $0.46 \pm 0.1$  to  $0.48 \pm 0.1$   $\mu\text{mol/l}$  in  
143 postmenopausal women ( $p < 0.05$ ) (Figure 1B). The plasma S1P/apoM ratio was not affected by training  
144 ( $p > 0.05$ ) (Figure 2A). As previously reported (40), plasma TC ( $p = 0.01$ ) and LDL-C ( $p < 0.01$ ) concentrations  
145 decreased after the training period, whereas plasma HDL-C and triglyceride concentrations did not change  
146 ( $p > 0.05$ ) (Table 1). The plasma apoM/HDL-C ratio was increased from  $0.50 \pm 0.1$  to  $0.54 \pm 0.2$  in  
147 premenopausal women and from  $0.56 \pm 0.2$  to  $0.60 \pm 0.2$  in postmenopausal women, which was borderline  
148 statistically significant ( $p = 0.05$ ) (Figure 2B).

149 *Correlations between apoM and lipids in plasma* - The plasma apoM concentration correlated positively  
150 with plasma TC, HDL-C, and LDL-C concentrations both before and after the training intervention, while no  
151 correlation was found between the plasma apoM and triglyceride concentration (Figure 3). Also, there was

152 no correlation between plasma apoM and S1P concentrations ( $p>0.05$ ) (Figure 4). Additionally, plasma S1P  
153 concentrations did not correlate with plasma TC, HDL-C, LDL-C, or triglyceride concentrations ( $p>0.05$ )  
154 (data not shown).

## 155 Discussion

156 There were two main findings in the present study. Firstly, recent postmenopausal women had higher  
157 plasma apoM than late premenopausal women. Secondly, exercise training increased plasma apoM and S1P  
158 in both late premenopausal and recent postmenopausal women.

159 Recent postmenopausal women had 32 % higher plasma apoM concentrations compared with late  
160 premenopausal women before the training intervention. There are at least two possible explanations for this.  
161 The first possible explanation for the higher postmenopausal apoM concentration is a difference in lipid  
162 levels. The postmenopausal women had higher TC (by 15 %), HDL-C (by 21 %), and LDL-C (by 14 %)   
163 compared with the premenopausal women before the training intervention. In accordance with previous  
164 findings, apoM correlated positively with plasma TC, HDL-C, and LDL-C (1). ApoM also correlates  
165 negatively with the fractional catabolic rate of LDL (9), suggesting that LDL-C should be increased when  
166 apoM is increased, which is consistent with our findings. As discussed previously (31), there is a general  
167 agreement that TC and LDL-C are elevated in postmenopausal women compared with premenopausal  
168 women, rendering a more atherogenic profile (7, 14, 25). In contrast, findings on the relationship between  
169 menopause and HDL-C and triglycerides are inconsistent (7, 14, 15, 25, 38). The Study of Women's Health  
170 Across the Nation (SWAN) found that HDL-C was higher in recent postmenopausal women ( $\leq 24$  months  
171 after the final menstrual period [FMP]), but then declined to the premenopausal level in late postmenopausal  
172 women ( $> 24$  months after the FMP) (14). In the present study, no significant correlation between lipid levels  
173 or apoM levels and age was found (data not shown). A study on a sub-cohort from SWAN also found that an  
174 increase in HDL-C over the menopausal transition was associated with a greater development of  
175 atherosclerosis (17). This is an interesting observation as HDL particles are generally considered anti-  
176 atherogenic (20). The finding that plasma apoM was higher in the postmenopausal women might be  
177 explained by a concomitant increase in HDL-C, illustrated by a stable apoM/HDL-C ratio between pre- and  
178 postmenopausal women. Thus, one may speculate that apoM-containing HDL particles may lose their  
179 endothelium-protective and anti-atherogenic potential over the menopausal transition. Nyberg et al., found  
180 that the early postmenopausal phase was associated with a marked reduction in vascular function in the  
181 Copenhagen Women Study (37), and observed that several biomarkers of vascular function were adversely

182 altered in a similar cohort (38). Further studies are however still needed to conclude whether apoM-  
183 containing HDL plays a role in this reduction. Second, it is possible that the higher apoM concentration can  
184 be explained by the changes in hormone levels that occur over the menopausal transition and possibly even a  
185 direct effect of sex hormones on apoM. *Axler et al.* found that apoM concentrations correlate positively with  
186 age for women only, with women aged 18-49 years having lower apoM concentrations than women aged 50  
187 years or older (1). This observation does not prove a link between apoM and menopause, but the study  
188 supports the notion that apoM concentrations could be dependent on hormone levels, as the positive  
189 correlation with age is seen for women only. Few studies have addressed the effect of sex hormones on  
190 apoM, but it has been shown that estrogen upregulates apoM expression in vitro and in vivo in rats (44). This  
191 suggests that plasma apoM concentrations should be higher in pre- than postmenopausal women, which was  
192 not the case in the present study. Further, we did not find any correlation between levels of estrogen and  
193 apoM (data not shown). Thus, present findings suggest that the difference in apoM levels between groups  
194 may not be related to estrogen alone, but rather to a combination of age, hormonal status, and other unknown  
195 variables.

196 Plasma S1P was similar in pre- and postmenopausal women, causing the S1P/apoM ratio to be  
197 significantly lower in postmenopausal women. A recent study found that apoM without S1P did not have  
198 anti-inflammatory properties (41); this further supports the notion that the higher apoM concentrations in  
199 postmenopausal women do not necessarily provide an atheroprotective effect as the S1P concentration did  
200 not differ between the two groups. This finding is in contrast to an earlier study which found that plasma S1P  
201 in premenopausal women. The study found S1P to be negatively correlated with age in both men and women  
202 (22). A disadvantage of the study is a large age difference between the subjects (~30 years), and lack of  
203 follow up on the same subject before, during, and after postmenopausal transition. While the Copenhagen  
204 Women Study neither is a prospective study, it has the strength of a minimal age difference (~4 years)  
205 between the two groups. In the future, it would be relevant to examine the effect of menopause on the  
206 apoM/S1P axis by following the same cohort throughout the menopausal transition since the pre-menopausal  
207 women in the present study could be at varies pre-transitional ages.

208           The training intervention increased plasma apoM and plasma S1P. Importantly, the S1P/apoM ratio  
209   did not change in contrast to the menopause-related changes, implying that the training-induced increase is  
210   different from and possibly renders a more atheroprotective profile than the menopause-induced changes.  
211   There are at least two possible explanations for the post-training increase in apoM. First, it is possible that  
212   the exercise-induced changes can be explained by changes in plasma lipids. The general consensus is that  
213   exercise training increases HDL-C in healthy adults, providing an atheroprotective effect (13, 29). However,  
214   previous findings in postmenopausal women have shown that exercise training decreases TC and LDL-C  
215   without changing HDL-C (4), which is in accordance with the present findings. Thus, it does not seem likely  
216   that the post-training increases in apoM and S1P can be explained solely by changes in plasma lipids.  
217   Another possible explanation could be that training also lowered plasma insulin during the oral glucose  
218   tolerance test (31) which could lead to an increase in plasma apoM as insulin inhibits the expression of apoM  
219   through a Foxa2-mediated mechanism (46). The increase in S1P with training agrees with a previous study  
220   showing that plasma S1P was 37 % higher in endurance trained (average experience of  $4.3 \pm 1.7$  years of long  
221   distance running) than in untrained healthy, young males (2). However, another study found no difference in  
222   plasma S1P between endurance-trained athletes and obese, sedentary controls (3). The reason for this  
223   discrepancy between studies is unclear, but the variation may be due to differences in study setups, including  
224   gender, age, and duration of the training period.

225   *Potential Clinical Value* – Currently, there is a lack of mechanistic understanding of changes that occur  
226   during the menopausal transition. Also, few intervention studies have been conducted addressing the effects  
227   on plasma apoM levels in humans. One study has shown that 8 weeks of statin treatment decreases apoM by  
228   7% (26). In the present study the range of changes are comparable observed between pre-and  
229   postmenopausal women. However, the higher apoM in postmenopausal women without a concomitant  
230   increase in S1P may contribute to understanding how previously atheroprotective apoM-containing HDL  
231   particles can lose their anti-atherogenic and endothelium-protective potential in the menopausal transition.  
232   The finding that postmenopausal women have a lower S1P/apoM ratio gives rise to the question of whether  
233   S1P analogues – which are currently released on the market for treatment of multiple sclerosis (43) – can be

234 beneficial in treating risk factors for endothelial dysfunction and atherosclerosis related to the  
235 postmenopausal phase. Finally, exercise increased both plasma apoM and S1P in pre-and postmenopausal  
236 women. It is likely that an extended period of training could increase the plasma apoM/S1P levels further. To  
237 maintain a high level of apoM – and S1P containing HDL particles could be of clinical value due to its anti-  
238 atherogenic and endothelial protective value.

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378 **Figure legends**

379 **Figure 1. Effect of menopausal status and exercise training on plasma apoM and S1P concentrations.**

380 (A) Plasma apoM concentrations in pre- (n=36) and postmenopausal (n=37) women. Changes between  
381 groups were analyzed using Two-way repeated measures ANOVA: menopause\*exercise,  $P>0.05$ ; effect of  
382 menopause,  $P<0.0001$ ; effect of training,  $P<0.05$ . (B) Plasma S1P concentration in pre- (n=30) and  
383 postmenopausal (n=30) women. Changes between groups were analyzed using Two-way repeated measures  
384 ANOVA: menopause\*exercise,  $P>0.05$ ; effect of menopause,  $P>0.05$ ; effect of training,  $P<0.04$ . Only  
385 women with measurements before and after training have been included. \* $P<0.05$ : significantly different  
386 from premenopausal. # $P<0.05$ : significantly different from before training. ApoM, apolipoprotein M; S1P,  
387 sphingosine-1-phosphate.

388 **Figure 2. Effect of menopausal status and exercise training on plasma S1P/apoM and apoM/HDL-C**

389 **ratios.** (A) Plasma S1P/apoM ratio in pre- (n=30) and postmenopausal (n=30) women. Changes between  
390 groups were analyzed using Two-way repeated measures ANOVA: menopause\*exercise,  $P>0.05$ ; effect of  
391 menopause,  $P<0.001$ , effect of training  $P>0.05$ . (B) Plasma apoM/HDL-C ratio in pre- (n=34) and  
392 postmenopausal women (n=36). Changes between groups were analyzed using Two-way repeated measures  
393 ANOVA: menopause\*exercise,  $P>0.05$ ; effect of menopause,  $P>0.05$ , effect of training  $P=0.05$ . Only  
394 women with measures before and after training have been included. \* $P<0.05$ : significantly different from  
395 premenopausal. ApoM, apolipoprotein; S1P, sphingosine-1-phosphate; HDL-C, high-density lipoprotein  
396 cholesterol.

397 **Figure 3. Linear correlation between plasma apoM and lipid concentrations.**

398 Correlations between  
399 plasma apoM and TC (A, B), HDL-C (C, D), LDL-C (E, F), and triglyceride (G, H) concentrations in all  
400 women before training (n=73) and after training (n=72). Data were evaluated by Pearson's correlation.  
401 ApoM, apolipoprotein; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-  
density lipoprotein cholesterol.

402 **Figure 4. Linear correlations between plasma apoM and S1P concentrations.** (A) Pre- and  
403 postmenopausal women before training (n=68). (B) Pre- and postmenopausal women after training (n=64).  
404 Data were evaluated by Pearson's correlation. ApoM, apolipoprotein M; S1P, sphingosine-1-phosphate.

405 **Tables**

406 **Table 1: Lipid levels (modified from Mandrup et al. (40))**

	Premenopausal		Postmenopausal	
	Before training (n=36)	After training (n=36)	Before training (n=37)	After training (n=36)
<b>TC (mmol/l)</b>	4.89±0.7	4.75±0.7#	5.64±0.7*	5.48±0.6*#
<b>HDL-C (mmol/l)</b>	1.65±0.4	1.70±0.4	2.00±0.4*	2.03±0.4*
<b>LDL-C (mmol/l)</b>	2.88±0.7	2.72±0.6#	3.26±0.6*	3.17±0.6*#
<b>TRIG (mmol/l)</b>	0.83 (0.6-1.1)	0.86 (0.7-1.1)	0.85 (0.7-1.1)	0.78 (0.7-1.1)

407 Parametric data are given as mean±SD, and non-parametric data are given as median (25-75 percentiles).

408 Data are for all available measurements. Changes between groups were analyzed using Two-way repeated

409 measures ANOVA. Only women with measurements before and after training were included in the two-way

410 repeated measures ANOVA. No significant interactions for menopause\*exercise were found (P>0.05) for

411 any parameter. \*P<0.05: significantly different from premenopausal. #P<0.05: significantly different from

412 before training. TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density

413 lipoprotein cholesterol; TRIG, triglycerides.

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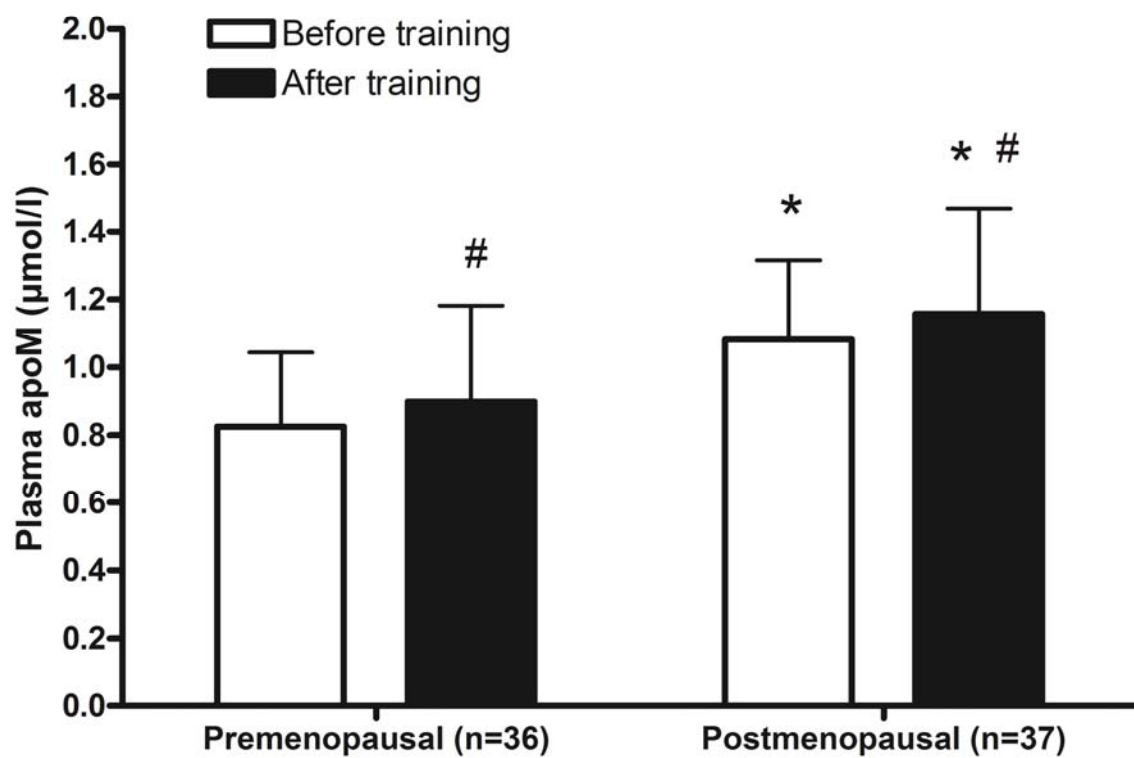
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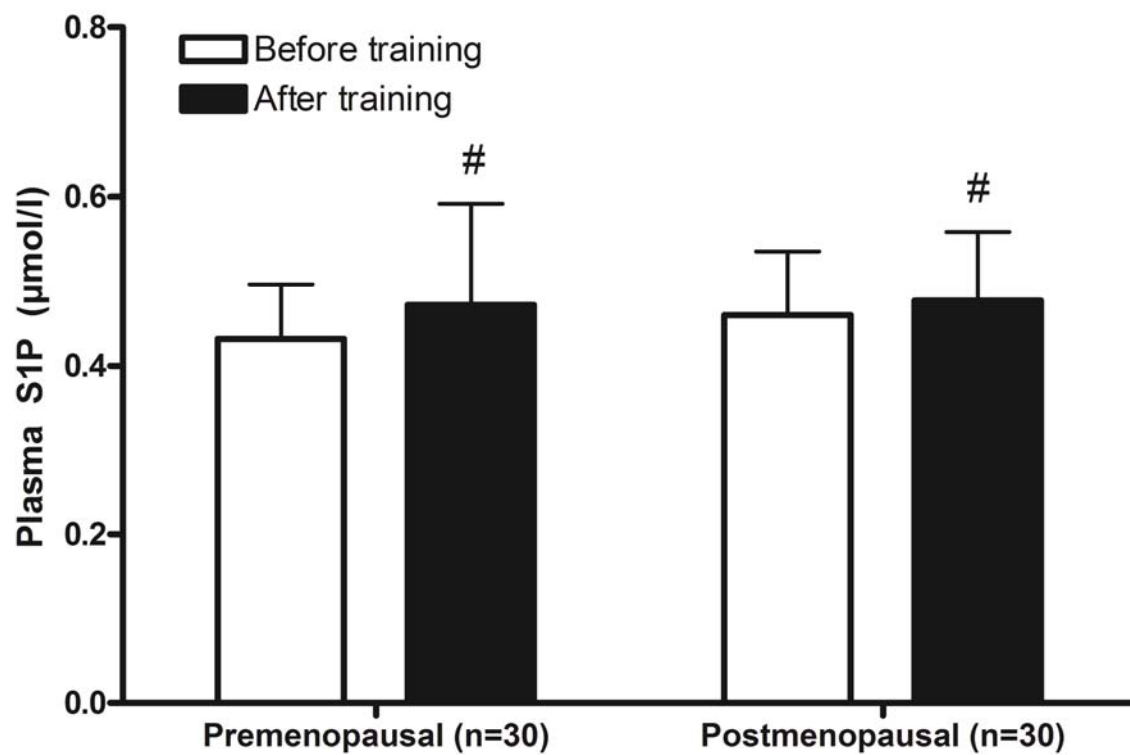
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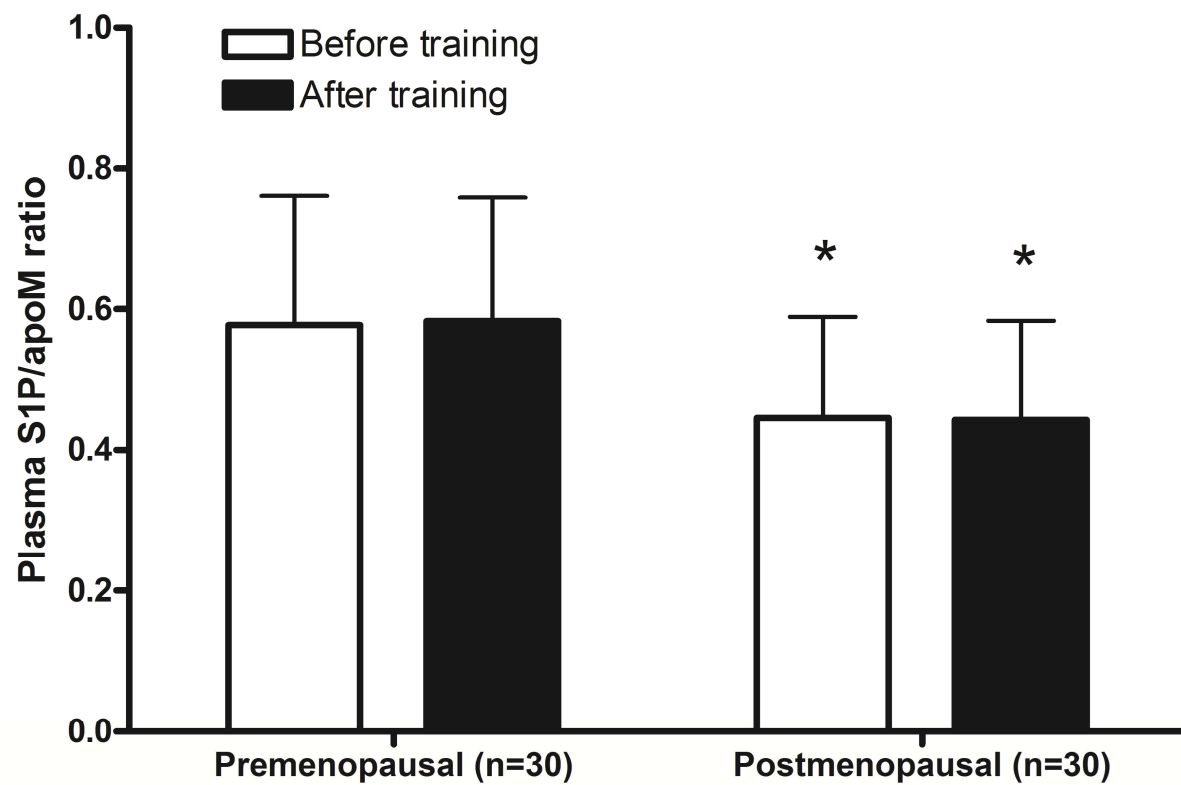
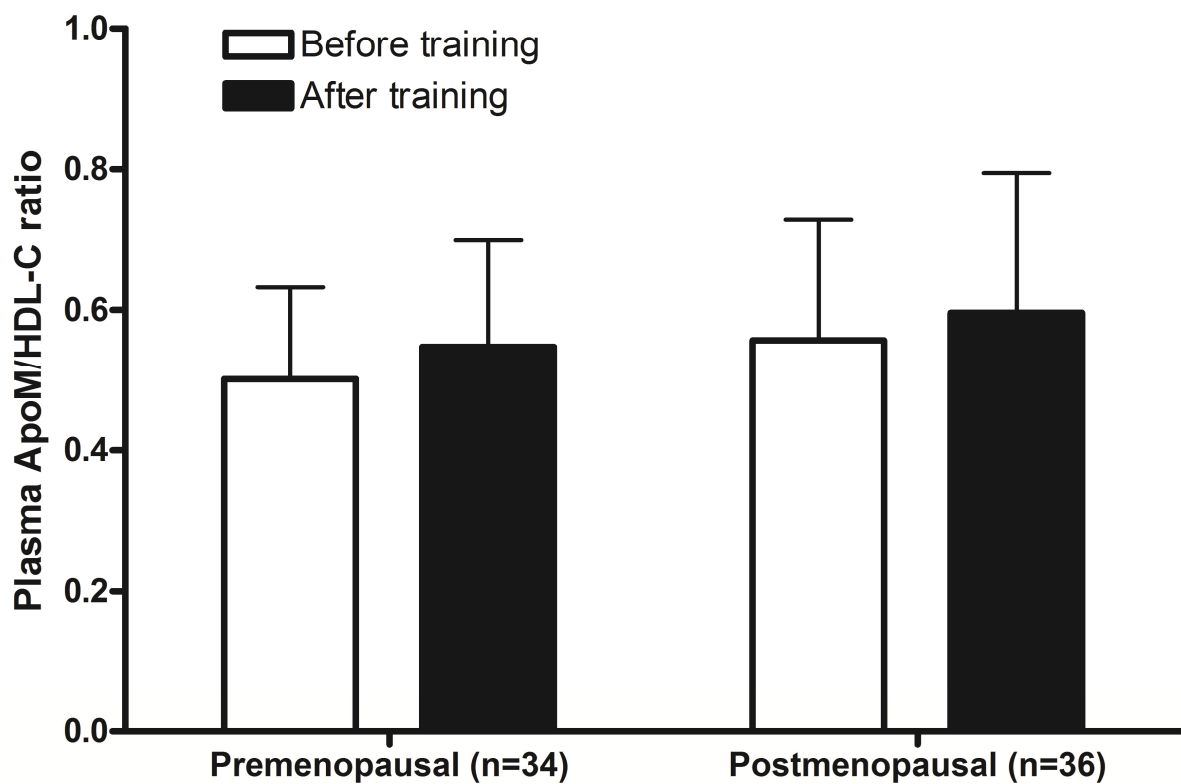
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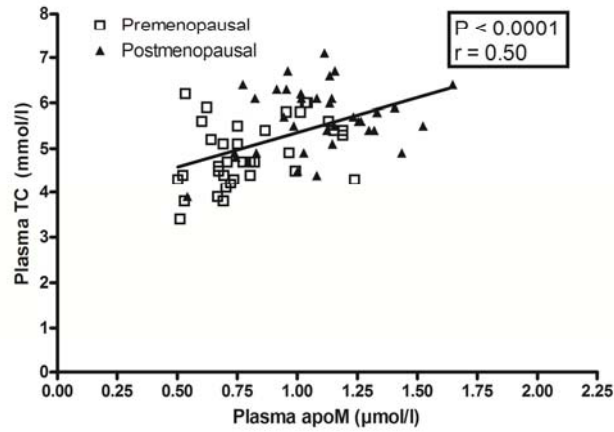


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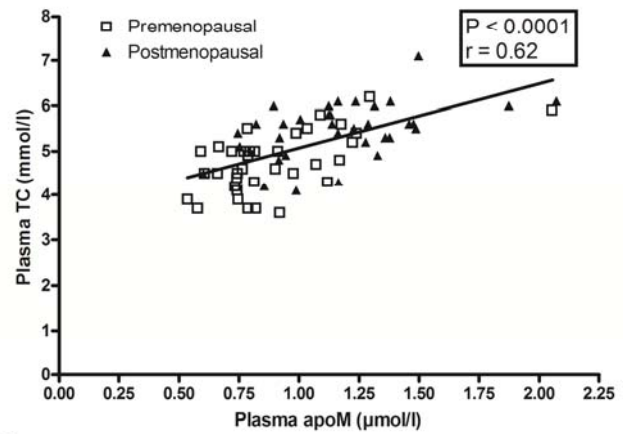
Before training (n=73)

After training (n=72)

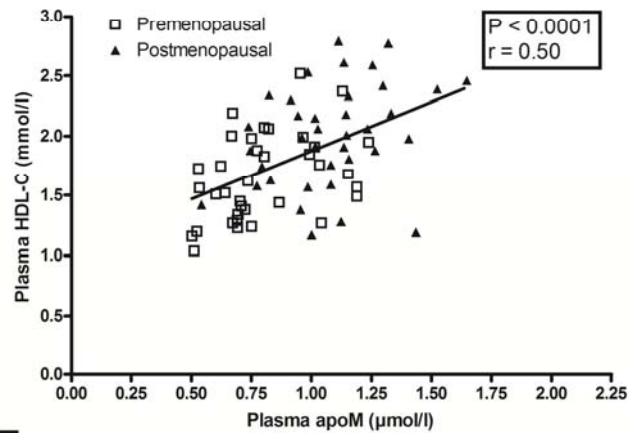
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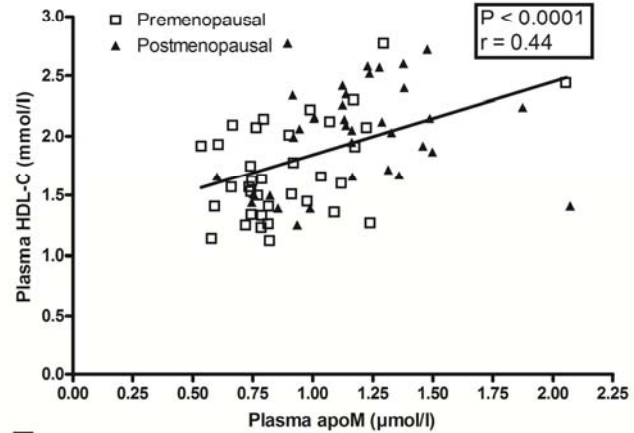
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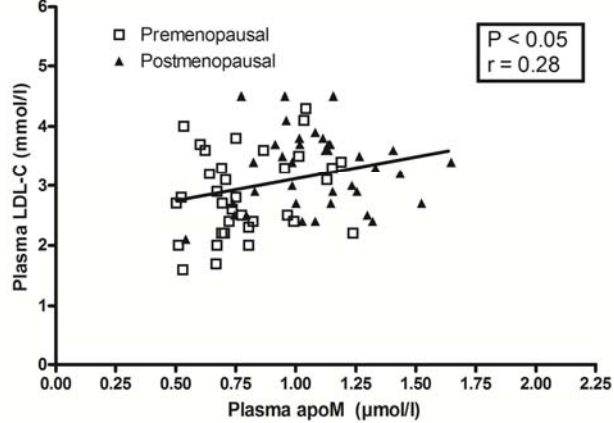
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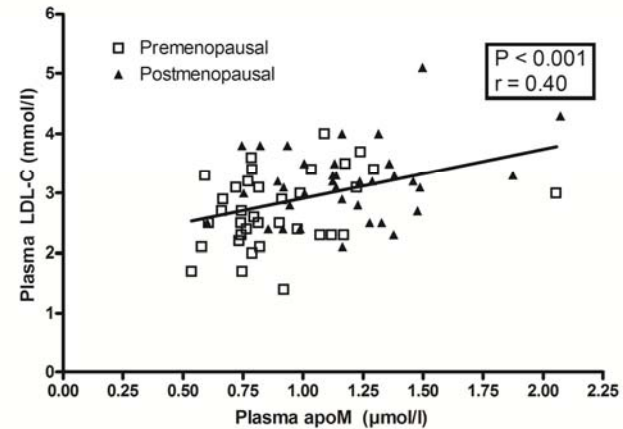
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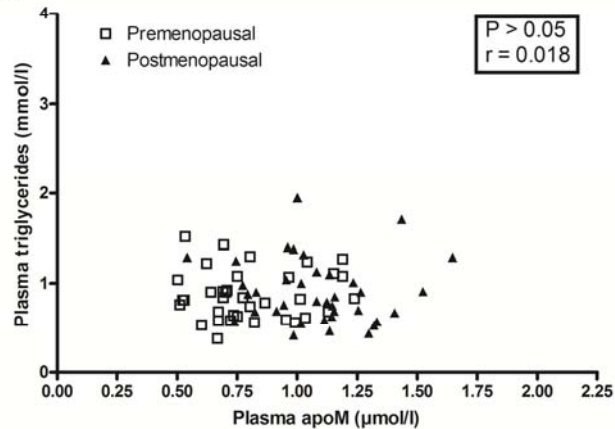
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F



G



H

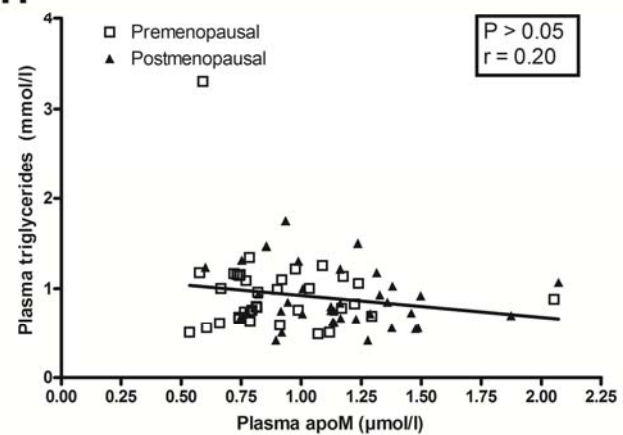
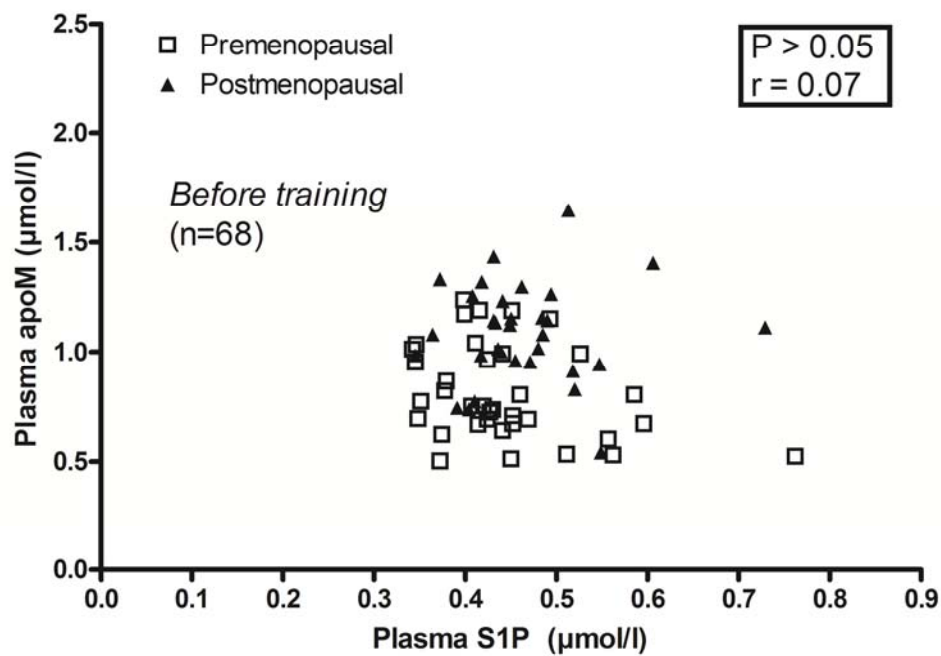
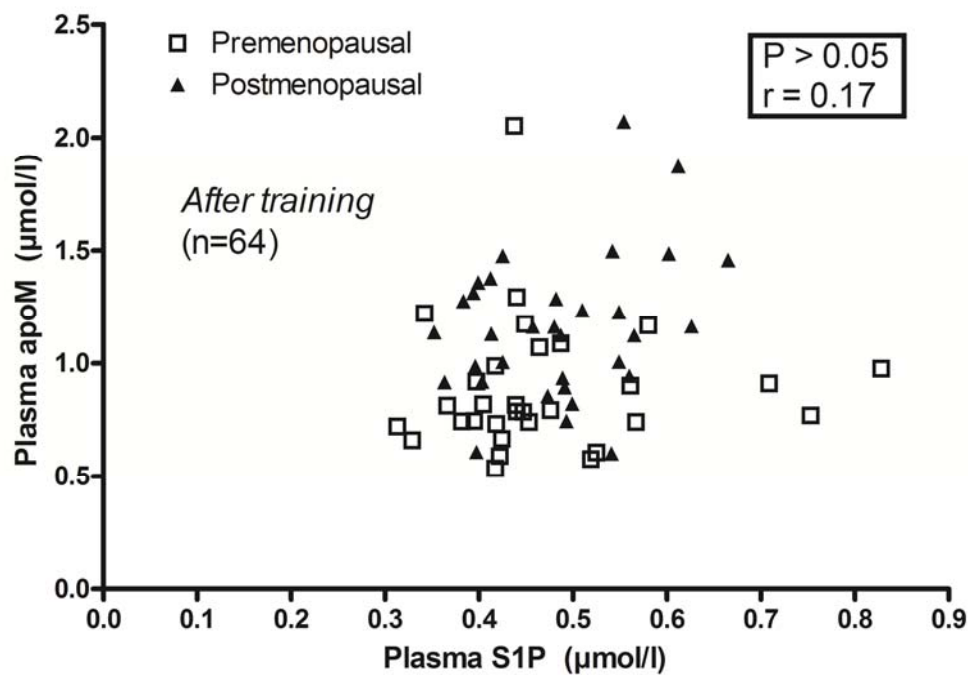


Figure 3, Yafasova et al.

**A**



**B**



**Figure 4. Yafasova et al**